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Engineered Photoactivatable Genetic Switches Based on the Bacterium Phage T7 RNA Polymerase

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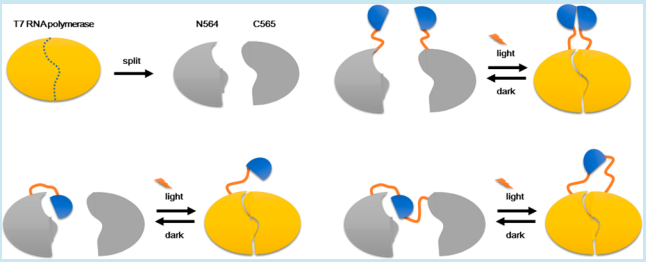
Supporting Information

ABSTRACT: Genetic switches in which the activity of T7 RNA polymerase (RNAP) is directly regulated by external signals are obtained with an engineering strategy of splitting the protein into fragments and using regulatory domains to modulate their reconstitutions. Robust switchable systems with excellent dark-off/light-on properties are obtained with the light-activatable VVD domain and its variants as regulatory domains. For the best split position found, working switches exploit either the light-induced interactions between the VVD domains or allosteric effects. The split fragments show high modularity when they are combined with different regulatory domains such as those with chemically inducible interaction, enabling chemically controlled switches. To summarize, the T7 RNA polymerase-based switches are powerful tools to implement light-activated gene expression in different contexts. Moreover, results about the studied split positions and domain organizations may facilitate future engineering studies on this and on related proteins.



KEYWORDS: *genetic switch, split T7 RNA polymerase, light-regulated protein−protein interaction, allosteric control, modular domain organization*

INTRODUCTION

Genetic switches are widely used as basic parts in synthetic biology, especially in the implementation of gene circuits. These switches allow gene expression conditioned on one or more endogenous or exogenous signals.[1,2] On the other hand, bacteriophage RNA polymerases such as the T7 RNA polymerase (T7 RNAP) have been extensively used for gene expression in prokaryotic[3,4] and eukaryotic[5] cells as well as in certain *in vitro* contexts.[6] These external RNA polymerases have the advantages of being orthogonal to a number of host machineries, such as promoter sequences and certain types of transcriptional regulations, and they often function robustly. To enable conditional gene expression by such a polymerase, one usually needs to use an extra upstream genetic switch that controls the gene expression of the RNA polymerase itself. If the protein activity of the RNA polymerase can be switched on or off directly, this extra level of control on the expression of the polymerase's own gene transcription will not be necessary, or in more complicated gene circuits, it can be used for other purposes. More importantly, RNA polymerase will then become a genetic switch itself. With the aforementioned advantages, such polymerase-based switches can be molecular parts of general use in synthetic biology.

In the current work, we investigate endowing T7 RNAP with switchable activity through protein engineering. Comprising only a single polypeptide chain, this polymerase is capable of transcribing RNA in abundance upon the highly specific recognition of a short 17-bp T7 promoter sequence.[3,7] The structure of T7 RNAP in complex with the promoter DNA has been reported,[8,9] providing important and general guidance for protein engineering. However, so far, only a few engineering-related studies on this widely applied protein have been attempted.[10−13] It has been found that the two separated peptide fragments of T7 RNAP split at amino acid position 179−180, when coexpressed, can reconstitute autonomously to regain gene transcription activity.[13] Later on, Segall-Shapiro et al. used a transposon-based method to find several other split positions of a similar property.[12] Depending on the split position, reconstitution may bring back varied levels of activity. The authors further showed that by fusing the split fragments to the paired protein−protein interaction domains SZ17 and SZ18, respectively, or by covalently rejoining the fragments through fusion with intein sequences, the reconstituted enzyme can reach the same level of activity as the wild-type T7 RNAP.[11,12] These results suggested that creating T7 RNAP-based genetic switches through, for example, splitting the enzyme into fragments and regulating the interactions between the fragments is viable. However, direct turning on/turning off of protein activity by an external signal has not been reported except for one study, which made use of a photoreactive non-natural amino acid.[10]

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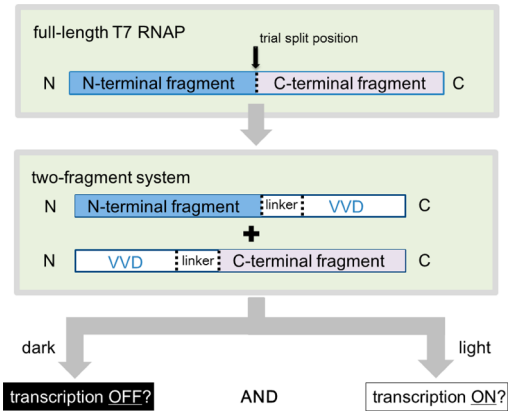
Among the various possible signals for the desired polymerase-based genetic switch to sense, light is of particular interest. In recent years, through the introduction of the genetically encoded light-sensitive proteins, optogenetics has become a powerful approach to manipulating the behavior of living cells and organisms by light.[14−16] Generally speaking, a light-responsive genetic switch can be a promising tool for the precise control of spatiotemporal gene expression in a variety of organisms. Engineering novel light-sensitive proteins, including genetic switches, has been a fruitful research field in the past decade. The aforementioned engineered light-responsive T7 RNAP[10] is a nice example. However, the tool relied on a pre-engineered host cell system that can introduce the non-natural amino acid, the caged tyrosine 1 (Tyr modified with an ONB group). This puts strong restraints on the applicability of such tools. A more widely applicable approach is to fuse a known light-sensitive protein domain to a target protein in a way so that one of the following two mechanisms could be exploited to achieve responsiveness to light.[17] One is through light-mediated allosteric effects. The other is through light-induced protein−protein interactions. For examples working by the allosteric mechanism, the light-sensing LOV (light-oxygen-voltage) domain has been utilized to successfully regulate various proteins, including the trp repressor[18] and the Rac1 protein,[19−21] and to induce protein colocalization or gene expression.[22−24] For examples based on protein−protein interactions, various studies have used the light-induced homodimeric interactions of the EL222 domain (a bacterial light-regulated DNA-binding protein) and of the VVD (fungal photoreceptor Vivid) domain;[25−27] or the light-induced heterodimeric interactions between *Arabiodopsis thaliana* PhyB (phytochrome B) and PIF3 or PIF6,[28,29] or those between GI (GIGANTEA) and FKF1 (the flavin-binding, kelch repeat, f-box1),[30,31] those between CRY2 (cryptochrome 2) and CIB1 (the cryptochrome-interacting basic-helix−loop−helix protein),[15,32−34] and those between Magnets.[35,36] The Magnets comprise a pair of VVD domain variants that have been rationally engineered to interact with each other specifically.[35]

The above studies suggested several candidate light-sensitive protein domains usable for engineering a light-sensitive T7 RNA polymerase that does not require non-natural amino acid. We have eventually used the VVD domain from the filamentous fungus *Neurospora crassa*. Of only 150 amino acids, VVD is one of the smallest currently known photoreceptors. It can form homodimer rapidly in response to blue light, and it requires no exogenous cofactor in prokaryotic and eukaryotic cells for this function.[37−39] Because of these properties, VVD has been proposed as especially suitable for engineering photoswitches based on light-induced protein−protein interactions. Recently, Kawano et al. utilized the VVD domain to control the complementation of split-firefly luciferase fragments in response to blue-light, and they developed the VVD variant pair Magnets,[35] which have been used to enable optical control of Cas9 (CRISPR associated protein 9).[36]

To explore splitting-based engineering of T7 RNAP and to obtain signal-responsive T7 RNAP-based gene switches using the VVD domain, we adopted the strategy depicted in Scheme 1. In this strategy, the T7 RNAP is split into an N-terminal and a C-terminal fragment at a series of trial positions. For each split position, VVD is fused to both fragments, resulting in two fusion proteins, whose abilities to jointly transcribe a green fluorescence protein (GFPuv)[40] gene under the light and dark conditions, respectively, are compared. Based on the structure of T7 RNA, the trial positions have been selected to be in different solvent-exposed loops. It turned out that T7 RNAP has high plasticity for this type of engineering, with a significant number of split positions leading to differentiated transcriptional activity under light and dark conditions, respectively. Several positions demonstrated excellent light-on/dark-off property. For a position that yields the best result, we studied a number of variants of the original design to investigate the possible mechanism of coupling between light sensing and the recovery of polymerase activity. It turned out that, at this position, the split polymerase can be robustly regulated by either light-induced protein−protein interaction or light-induced allosteric effects. To test whether the combination between the split T7 RNAP fragments and the extra regulating domain is modular, we have also replaced the VVD domains by a pair of protein domains of chemically inducible interactions,[41] and we investigated the response of the resulting system to the corresponding chemical signal.

Scheme 1. Constructing a Two-Fragment System Based on Split T7 RNAP and VVD To Test if a Trial Split Position on T7 RNAP Leads to Switchable Polymerase Activity



RESULTS AND DISCUSSION

Light Responsiveness for Different Split Positions. Figure 1A and 1B show that among the nine split positions investigated, seven of them lead to differentiated GFP levels between dark and light conditions. For these seven positions, the GFP levels at the light state, all being significantly above the negative background, varied between different positions, with the position 564−565 leading to the highest GFP level, which was comparable to that of the wild-type T7 RNAP. We then named the two-fragment system obtained by splitting T7 RNAP at position 564−565 and fusing both fragments to a VVD domain as paT7P-1 (paT7P stands for photoactivatable T7 RNA polymerase). At the dark state, all seven positions except position 179−180 led to GFP levels comparable to the negative background. For position 179−180, there was still a significant residual GFP level at the dark state, although it was notably lower than the level at the light state.

Some of the split positions studied here have been covered in previous studies which reported split positions that could regain partial polymerase activity through coexpression of the "pure" split fragments (i.e., fragment without any attached extra domains). These included, besides the position 179−180,[13] three other positions (positions 167−168, 564−565, and 600−601) that were covered by the previously reported seams.[12] With VVD domains fused to the split fragments, only position 179−180 led to polymerase activity in the dark state (although

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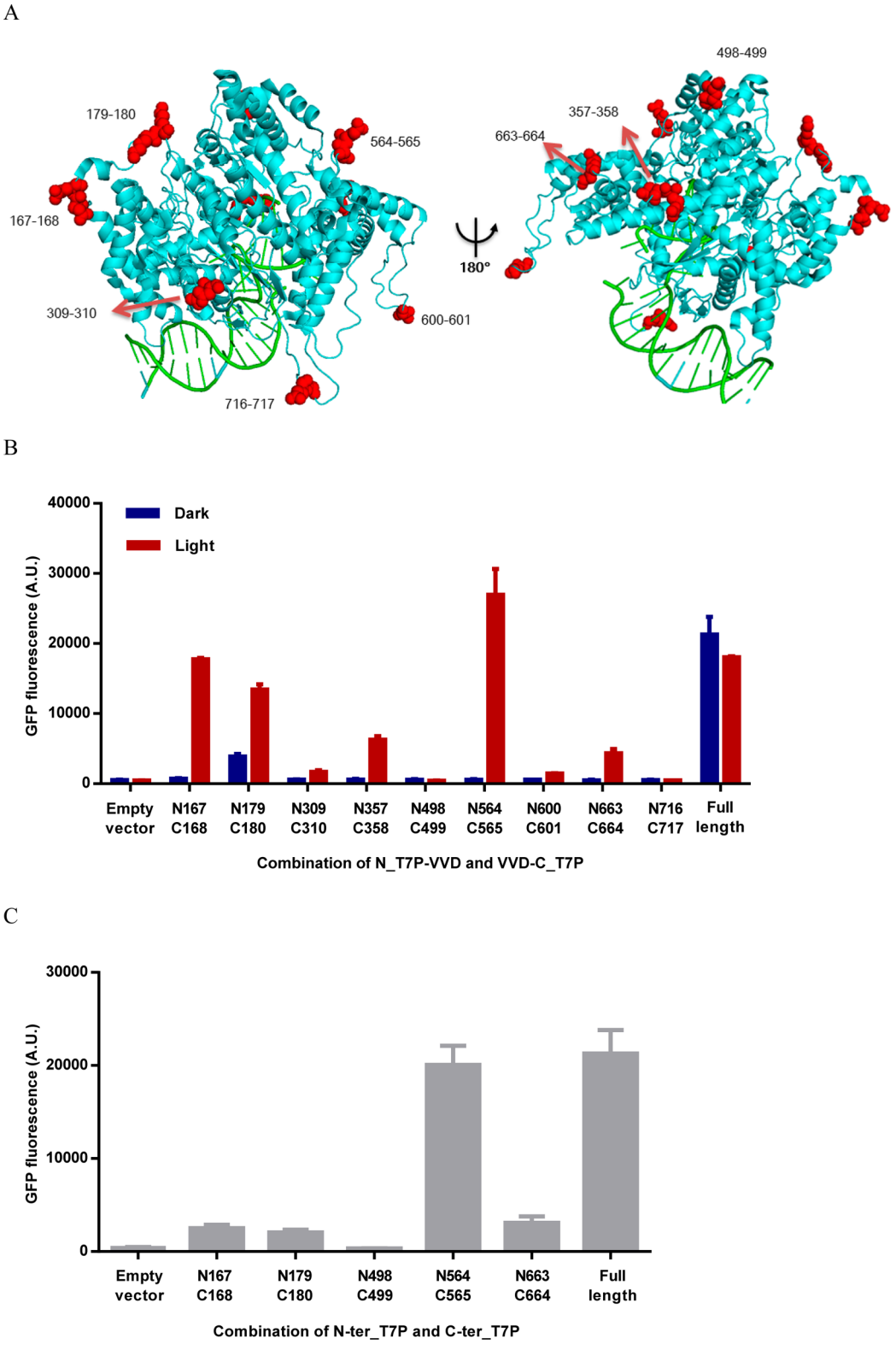

Figure 1. (A) The nine tested split positions (red spheres with labels of position numbers) are located on solvent-exposed loops of the structure of T7 RNAP (PDB ID: 1QLN). (B) Fluorescence strengths of the two-fragment systems associated with different split positions. A VVD domain is fused to each of the N- and C-terminal fragments. The dark and light conditions are compared. The horizontal axis is labeled by the participating fragments. In this and subsequent figures, the split N- (C-) terminal fragments are named with the letter N (C) followed by the respective ending (starting) amino acid positions. (C) Fluorescence associated with the paired "pure" fragments (i.e., without attached regulatory domains) associated with different split positions. "Empty" represents *E. coli* containing only the GFPuv-reporter. "Full-length" represents *E. coli* containing wild-type full-length T7 RNAP and GFPuv-reporter. In this and subsequent figures, fluorescence strengths are shown as the mean ± s.e.m. from three independent measurements.

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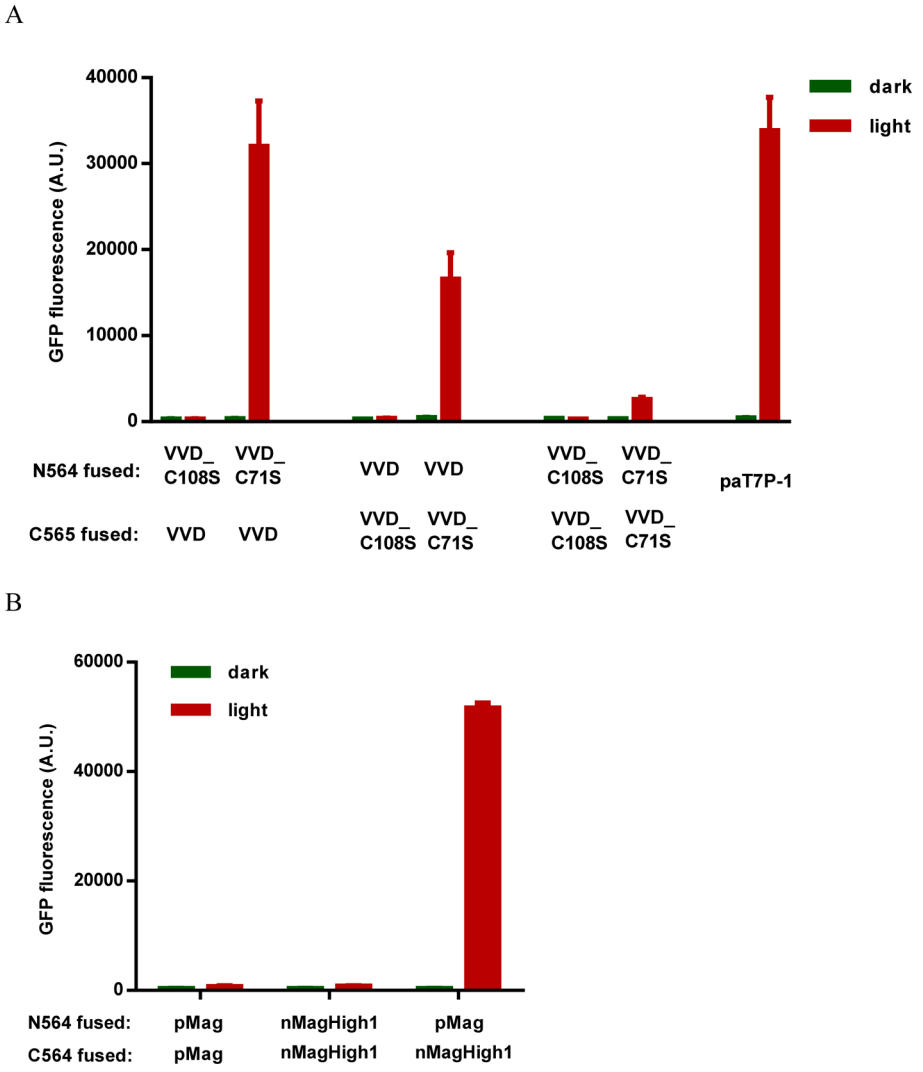

Figure 2. (A) Fluorescence strengths associated with various VVD_C71S and VVD_C108S mutants of paT7P-1. (B) Florescence strengths associated with the revised paT7P-1 systems, in which the VVD domains have been substituted by pMag or nMagHigh1. The horizontal labels indicate the paired fragments and the VVD or VVD variants to which they attach.

relatively weak), while the other three positions all led to no detectable activity in the dark state, suggesting that, in the dark state, the noninteracting regulatory domains strongly interfere with the reconstitution between the split fragments. At the light state, these positions led to active reconstitution, presumably because of the light-induced interactions between the regulatory domains.

Although all but split position 179−180 led to no detectable transcription activity in the dark state; only split positions 498−499 and 716−717 still led to no detectable activity at the light state. Among the remaining split positions (positions 167−168, 179−180, 309−310, 357−358, 564−565, 600−601, and 663−664) that led to intermediate to strong light-on activity, positions 309−310, 357−358, and 663−664 have not been reported by Segall-Shapiro et al.[12] We have (re)investigated the ability of the "pure" fragments to reconstitute into an active enzyme by removing the extra regulatory domains from the split fragments for positions 167−168, 179−180, 498−499, 564−565, and 663−664. The results are shown in Figure 1C. Only for position 498−499 were the "pure" fragments not able to reassemble into an active enzyme. For split fragments fused with VVD domains, the split position 498−499 also happened to lead to no detectable activity under the light condition. For all other positions, coexpression of the "pure" fragments led to various levels of GFPuv transcription. Among them, the split position 663−664 has not been found in the previous profiling effort.[12] Especially for position 564−565, the activity is detected to be as high as that of the nonsplit full-length T7 RNAP. The split position 564−565 happened to also lead to the strongest light-induced activity. These results together with the results of ref12 suggest that, for introducing extra regulatory domains into an existing protein, it may be an efficient strategy to choose split sites for which the resulting "pure" fragments (i.e., fragments without any fused extra domains) can autoreassemble into an active protein, although the goals of regulation involve significant or even complete loss of activity under at least one regulatory condition (here the dark state).

We note that the apparent activity of the split T7 RNAP N564−C565 with fused VVD domains is even higher than the full-length T7 RNAP, while the corresponding "pure" split two-fragment system (i.e., without any fused VVD domains) does not exhibit such an increase in activity relative to the full length protein (Figure 1C). There could be two possible explanations for the apparent higher activity of the re-engineered system. One

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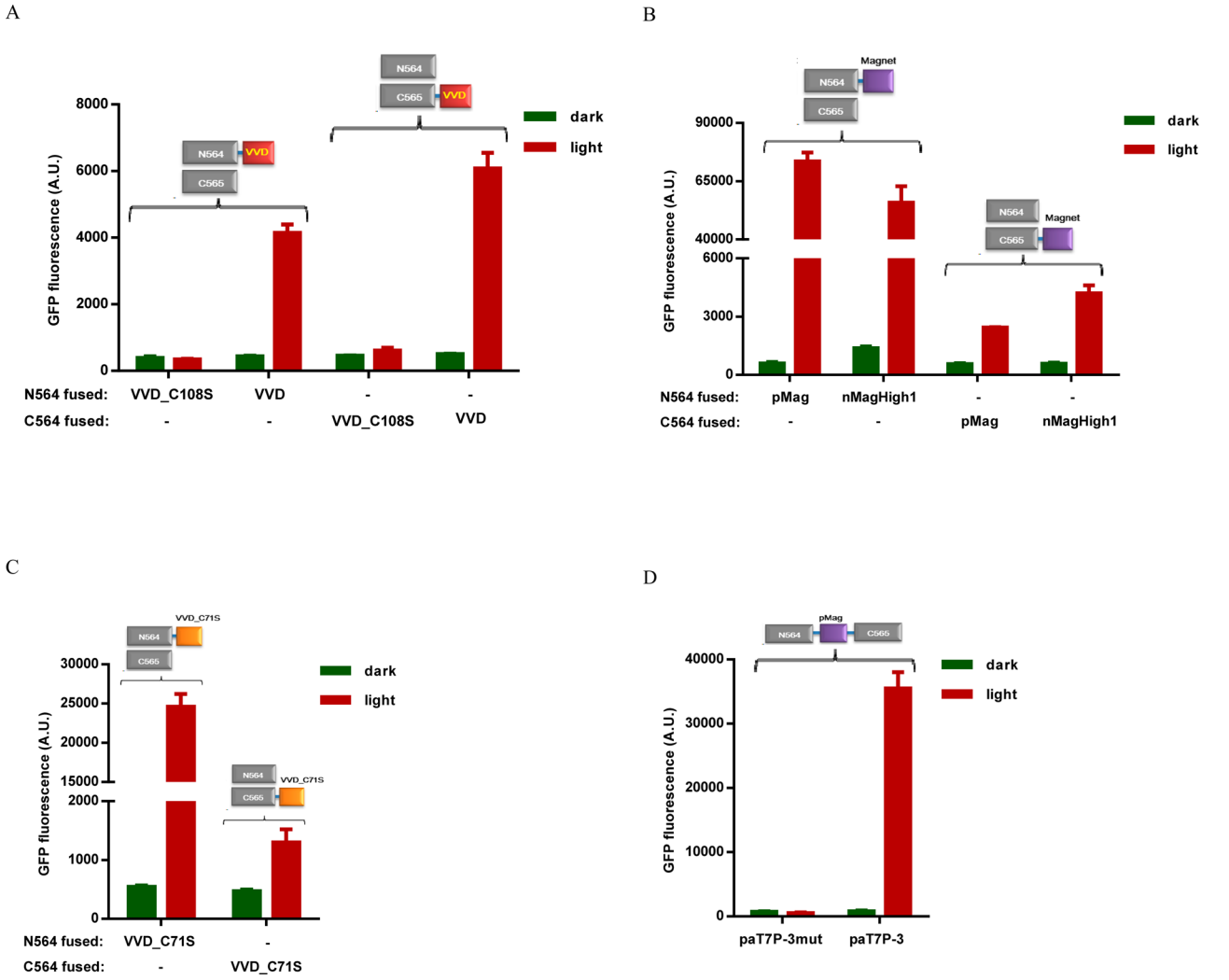

Figure 3. (A–C) Florescence strengths associated with the split T7 RNAP system with a single VVD or VVD variant domain attached to either the N- or C-terminal side of the fragments. (D) Fluorescence strengths associated with the paT7P-3 system, which is a full-length T7 RNAP with the pMag domain inserted at position 564–565. The paT7P-3mut system contains mutation pMag_C108S.

is that fusion to VVD domains may somehow increase the cellular protein level of the polymerase (for example, through increased protein stability). Another possibility, which is less likely but cannot be completely excluded, is that the engineered changes increase the intrinsic polymerase activity by coincidence.

Roles of Light-Induced Protein–Protein Interaction (PPI) in paT7P-1. Exposing to light may trigger a series of events for paT7P-1. The first is the photochemical reaction between the side chains of the active site Cys108 in each of the VVD domains and the bound FAD (flavin adenine dinucleotide) cofactor.[37,42] The chemical change may trigger conformational changes of the VVD domains, which may facilitate dimerization of the domains. This can be between the N-terminal side and the C-terminal side VVD domains (heterodimerization or N–C dimerization). Alternatively, it can be between two domains on the N-terminal side fragments themselves (N–N dimerization) or two domains on the C-terminal fragments themselves (C–C dimerization) (see Supplementary Figure 3A). Either the light-induced conformational changes of the VVD domains or the various light-induced PPI may eventually affect the attached T7 RNAP fragments, modulating the interactions between the latter and leading to activity change. To dissect possible roles of conformational change (allosteric) effects and of PPI, we constructed and analyzed a series of variants of the paT7P-1.

These variants comprised mutants of the VVD domain which have been known to impair light-induced conformational changes and/or subsequent dimerization. One mutant is C108S. It is experimentally known that the light-induced effects are triggered by the formation of a covalent bond between the side chain thiol group of C108 and the C4a of the cofactor FAD.[37,39] Thus, the C108S mutant of VVD will not be able to respond to light either through conformational change or dimerization.[27] Another mutant is C71S, Vaidya et al. have shown that this mutant is defected in forming homodimers in response to light.[39] It is, however, unclear whether the C71S mutant has completely lost the ability to respond to light in some other ways, for example, adopting conformational changes that do not lead to dimerization. Finally, a pair of VVD mutants engineered by Kawano et al.[35] have been used. They are pMag (positive Magnet) and nMag (negative Magnet). The actual domain used is nMagHigh1, which is nMag with M135I and M165I mutation,[35] which specifically forms heterodimer upon light exposure. As shown by Kawano et al., neither of these two domains forms homodimers. However, both pMag and nMag (or nMagHigh1) must have reserved their ability for light-induced

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conformational change, as conformational change is required for their heterodimerization.[35]

In a set of three variants, Cys108 in one or both of the VVD domains in paT7P-1 was mutated into serine (see Supplementary Figure 3B). The C108S mutations on either the N-terminal side VVD domain (N-VVD_C108S) or the C-terminal side VVD domain (C-VVD_C108S) alone, or on both VVD domains, led to the loss of the light-on function of the fragments (Figure 2A), confirming that the observed effects are the results of the photochemical reactions of VVD. In addition, for this particular construct, both VVD domains need to be photoreactive. As the corresponding "pure" T7 RNAP fragments without the extra VVD domains are active, the noninteracting dark-state VVD domains should have blocked the reconstitution of an active enzyme, perhaps through steric hindrance.

In another set of three variants, Cys71 of VVD has been mutated to serine (see Supplementary Figure 3B). Interestingly, although the variants containing mutations of C71S in the VVD domains on both sides lose their light-on functionality, both variants N-VVD_C71S and C-VVD_C71S, each containing a mutation in only one VVD domain on the respective N-terminal or C-terminal side, retain light-on activity (Figure 2A). A possible interpretation of these results is that the VVD_C71S, although it may not dimerize by itself, can still dimerize with a wild-type VVD under light conditions. We note from Figure 2A that N-VVD_C71S retains full activity while C-VVD_C71S only retains partial activity. This does not have a simple explanation and will be discussed later.

To further look into the role of the light-induced N−C dimerization in paT7P-1, we replaced the N-terminal side VVD domain with pMag and the C-terminal side VVD domain with nMagHigh1. The revised two-fragment system should have only light-induced N−C dimerization, but no light-induced N−N or C−C dimerization. This system retained the light-on/dark-off property of paT7P-1 (Figure 2B). On the other hand, with both VVD domains replaced by the same Magnets domain, pMag or nMagHigh1, the system loses light-on activity (Figure 2B). Results on these variants confirm that N−C dimerization is necessary for the paT7P-1 construct to be photoactivatable.

Roles of Allosteric Effects in Photoactivation of the Polymerase Function. The significantly different light-on activities of the N-VVD_C71S and the C-VVD_C71S variants of paT7P-1 (Figure 2A) suggested that, besides the simple mechanism of light-induced N−C dimerization, some other effects may also be affecting the system. In addition, although two extra VVDs in the dark state may cause sufficient steric hindrance to block the autonomous reconstitution between the T7 RNAP fragments, we wonder if a single extra VVD domain can do the same.

To look into these issues, the paT7P-1 system was reduced to contain only one VVD domain, which is attached to either the tail of the N-terminal side fragment (N-paT7P-1) or the head of the C-terminal fragment (C-paT7P-1). Figure 3A shows that, in the dark state, these two systems lack polymerase activity. Thus, a single extra VVD domain in its dark state is sufficient to inhibit the autonomous reconstitution. However, Figure 3A also shows that N-paT7P-1 has residual light-on activity, while C-paT7P-1 has intermediate light-on activity. Thus, either the N-terminal side or the C-terminal side VVD domain alone can realize light-induced activity recovery. The C108S mutants of both N-paT7P-1 and C-paT7P-1 lose light-on activity, confirming the involvement of the photochemical reaction (Figure 3A).

As light cannot induce N−C VVD dimerization in either N-paT7P-1 or C-paT7P-1, the light-on mechanism of these systems must be somewhat different from that of paT7P-1. In these systems, the effects of light, including light-induced conformational changes, may facilitate N−N or C−C dimerization besides the desired N−C dimerization. To retain only conformational change while abolishing N−N or C−C dimerization, their respective VVD domains were replaced by either pMag or nMagHigh1. The revised N-paT7P-1 systems show significantly increased light-on activity, while the revisions on C-paT7P-1 have some negative effects on the activity (Figure 3B). As neither of these VVD variants is capable of homodimerization, these results suggest that the turning on process of N-paT7P-1 and C-paT7P-1 by light is mainly through conformational change and allosteric effects instead of PPI. Besides this, the current data suggests that N−N dimerization caused by light-induced PPI may have a negative impact on the desired light-on activity: the N-paT7P-1 that contains the original VVD domain (which is capable of homodimerization) (Figure 3A) is significantly lower in activity than its revisions with the VVD domains replaced by the Magnets domains (which are not capable of homodimerization) (Figure 3B). In addition, the VVD_C71S mutant of N-paT7P-1, which has defects in N−N dimerization, also shows increased light-on activity as compared with N-paT7P-1 (Figure 3C). The retained light-on activity of the latter mutant also suggests that the C71S mutant is still capable of responding to light through conformational changes, although in a modified way that does not lead to dimerization. The current data also suggests that C−C dimerization might be beneficial to the activity of C-paT7P-1. The revised C-paT7P-1 containing the pMag or nMagHigh1 (Figure 3B) that lacks the ability of C−C dimerization has lower activity than the original C-paT7P-1 containing the original VVD (Figure 3A). In addition, the VVD_C71S mutant of C-paT7P-1 shows much impaired light-on activity (Figure 3C). We note that, in this latter mutant, not only C−C homodimerization has been impaired, but light-induced conformational changes may also have been modified. We also note that the trends caused by the C71S mutations in N-paT7P-1 and C-paT7P-1 (Figure 3C) are somewhat in parallel with the respective activity changes from paT7P-1 to its mutated variants N-VVD_C71S and C-VVD_C71S, respectively (Figure 2A).

The major role of allosteric effects instead of PPI in N-paT7P-1 and its revised systems suggests that splitting T7 RNAP into two fragments may not be necessary. A nonsplit system using the pMag domain with flanking linkers inserted at position 564−565 of T7 RNAP was constructed. This system also shows excellent light-on/dark-off activity that relies on the photochemical reaction (Figure 3D).

Time Courses of the on/off Process in Different Systems. From the above explorations, several systems have been obtained with good steady state switch property, which means strong transcriptional activity in the light state with undetectable transcriptional activity in the dark state. Besides the paT7P-1 two-fragment system, the revised N-paT7P-1 system with the VVD domain substituted by pMag performs the best among the different single VVD domain-containing two-fragment systems. We named this system paT7P-2. In addition, the single chain T7 RNAP with the pMag domain inserted at position 564−565 is named paT7P-3. According to discussions so far, paT7P-1 to 3 vary significantly in their underlying light-switching mechanisms, with paT7P-1 mainly through light-induced dimerization of the VVD domains attached to different

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fragments, paT7P-2 through light-triggered allosteric effects of one VVD domain on the reconstitution between T7 RNAP fragments, and paT7P-3 through light-triggered conformational changes of a single peptide chain. To investigate if these mechanistic differences affect the on/off dynamics, time dependent fluorescence strengths were measured for these systems during their turning on and turning off processes, and the results are shown in Figure 4.

For the speeds of turning on of all three systems, the transcription of GFPuv has already started before the first time point of the measurement (Figure 4). In addition, the increasing rates of GFPuv, which should correspond to the amounts of activated polymerase in the respective systems, kept increasing for several hours and peaked after 5−7 h (see Supplementary Figure 4A), which should be the time needed for full activation. Thus, under our experimental setup, the rate of activation as measured by one over the time needed for half activation ($1/t_{1/2}$) should be 0.3−0.5 per hour.

For the speeds of turning off, data in Figure 4 suggest differences between the three systems. For paT7P-1, the increase of fluorescence strength stopped approximately 2 h after the light was off, while for paT7P-2 and paT7P-3, the time is more than 3 h. This difference in turning off speed is more notable in Supplementary Figure 4B, in which the ratios of the fluorescence strength during the light−dark transition over the fluorescence strength in the constantly lighted condition are plotted as functions of time. The curves show that paT7P-1, which relies on light-induced N−C dimerization, is the fastest to turn off. The paT7P-2 and paT7P-3 have similar speeds of turning off, both being much slower than paT7P-1. If we assume the rate-limiting step for the turning off of the photoactivated systems to be the transition of the VVD domain from a lighted conformational state to a dark conformational state, a possible explanation for the faster turning off of paT7P-1 is that this system contains two VVD domains, and the enzyme can be turned off by either of the two domains making a transition after the light has been turned off, while both paT7−2 and paT7P-3 have only a single VVD domain, and their turning off requires the transition from the lighted-state to the dark state of this single domain.

Modularity of the Combination between the Split T7 RNAP Fragments and the Regulatory Domains. The role of light-induced PPI in the paT7P-1 system suggests that the combination between the respective T7 RNAP fragments and the regulatory domains can be modular. Namely, the VVD domains may be replaced by other protein domains with a switchable PPI function. This can lead to a genetic switch controlled by other signals. We tested this by substituting the VVD domains in paT7P-1 with the PPI domain pair FRB and FKBP12, whose dimerization can be induced by the small molecular rapamycin.[43] The system indeed demonstrated good switch properties controlled by rapamycin (Figure 5): without rapamycin, no detectable transcriptional activity was observed, while, with rapamycin added into the culture, the polymerase activity increased significantly. We note here this chemically inducible system has not been optimized with respect to domain organizations and linker sequences and so on.

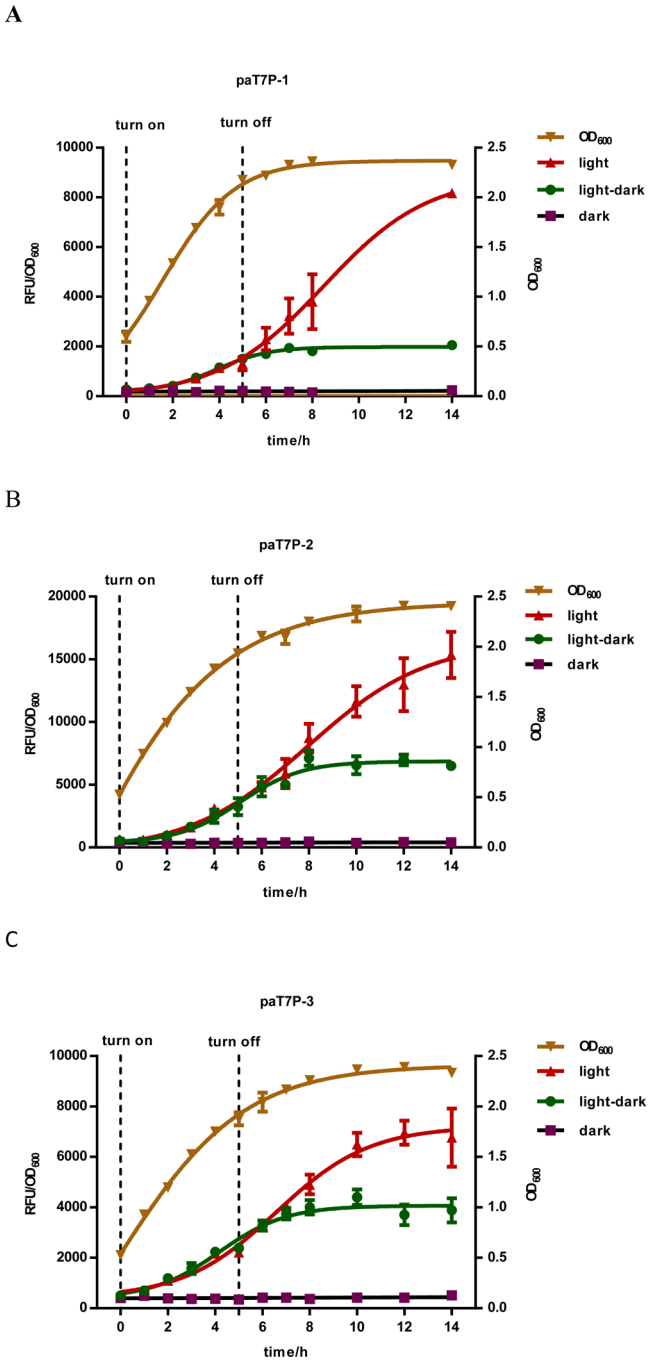

Figure 4. Fluorescence strengths changing with time for the different systems. The conditions include always dark (violet square), light-on at time zero and then remaining lighted (red triangle), and light on at time zero and then off after 5 h (blue circle). (A−C) correspond to systems paT7P-1 to paT7P-3, respectively. Growth curves for different systems are shown for the continuously lighted condition (orange inverted triangle). Curves for the other conditions are similar. Only for visual guidance, continuous lines fitted to data are drawn, with the fluorescence data for always dark fitted to straight lines, and the other data fitted to sigmoidal curves.

CONCLUSIONS

The T7 RNAP-based light-controlled genetic switches reported in this work can be used as optogenetic tools to control gene expression in different contexts. In general, they have excellent switch properties, including low leaky expression in the off state and high activity in the on state. They also have good reversibility, with varied switching off time that may suit different applications. Compared with the photocaged T7 RNAP reported before,[10] the current switches do not require any non-natural amino acid translation system, and they use blue light, which is much less toxic to cells than the UV light used to break the photocaged bond.

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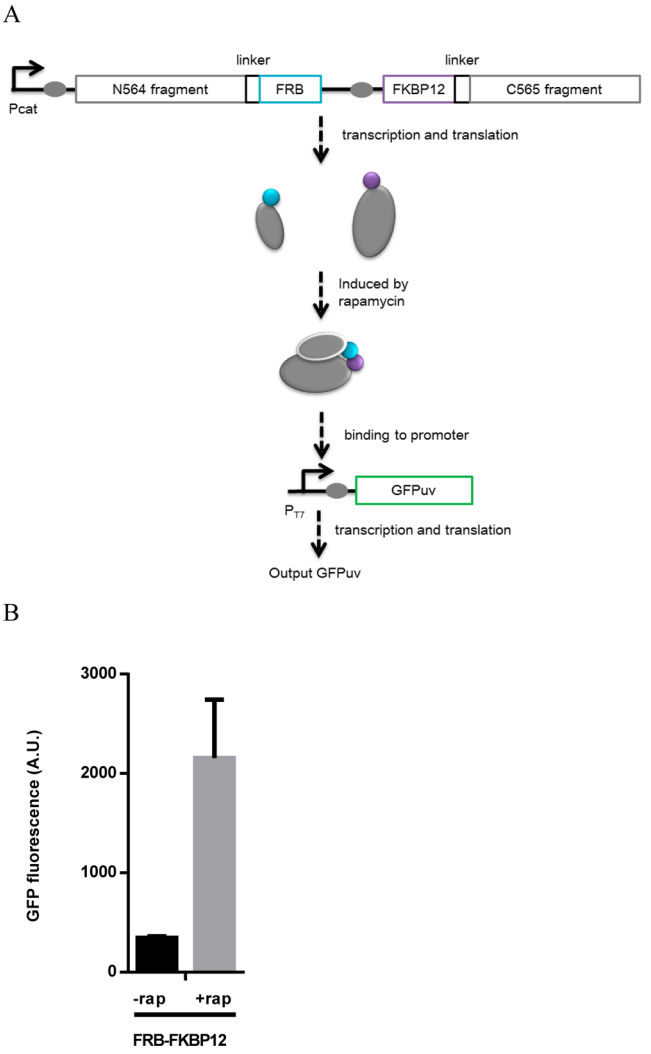

Figure 5. (A) Schematic depiction of the rapamycin-controlled two-fragment system based on splitting T7RNAP at position 564−565 and fusing the resulting N-terminal fragment to FRB and FKBP12 to the resulting C-terminal fragment. (B) Fluorescence strengths of the system without and with rapamycin.

Results and insights from the current study may facilitate future engineering efforts on T7 RNA polymerase and related proteins. The T7 RNAP shows high plasticity to succumb to the engineering strategy adopted in the current work, which is to create protein switches by splitting the enzyme into fragments and then using extra regulatory domains to modulate interactions between the fragments. The majority of split positions located in different solvent-exposed loops of T7 RNAP led to working switches. These positions may serve as candidate positions for splitting or insertion in other engineering efforts on T7 RNAP. For our case of using VVD as the regulatory domain, the split position 564−565 leads to the best results. As shown here for this split position and the VVD domain, mechanistically varied switches can be created based on the same split position and the same regulatory domain. Such analyses are not only good for understanding; they can be practically relevant as well, because mechanistic variations are often associated with variations in switching dynamics, as observed here. In addition, we have verified for the spit position 564−565 that modularity exists for the combination of the split T7 RNAP fragments with the extra regulatory domains. In the future, this modularity can be exploited to engineer polymerase switches controlled by signals other than the blue light or rapamycin signals tested here.

MATERIALS AND METHODS

Materials. Oligonucleotides and codon-optimized genes encoding N-terminally truncated VVD (VVD-36),[38] FKBP12 (the 12-kDa FK506-binding protein), and FRB (FKBP-rapamycin binding domain)[41] were synthesized by Sangon Biotech Co. (Shanghai, China) or General Biosystems Inc. (Anhui, China). Genes encoding pMag, nMagHigh1, and nMag were prepared as previously described.[35] The T7 RNA polymerase gene was amplified from the *E. coli* BL21 (DE3) strain. Restriction endonucleases and T4 DNA ligase were from Thermo Fisher Scientific Inc. (MA, USA). PrimeStar HS DNA polymerase was from TaKaRa Biotechnology Co. (Dalian, China). All chemical reagents were of analytical grade.

Bacteria were cultured with 1 × Luria-Bertani medium (LB: 10 g Bacto-tryptone, 5 g yeast extract, 10 g NaCl per 1 L), with appropriate antibiotics.

Restriction digests and ligations were performed with standard protocols. Chemically competent TOP10 cells were used for subcloning. Plasmids were purified using Miniprep Purification Kit II (GeneMark).

Plasmid Structures and Molecular Cloning. The T7 RNAP systems and the GFPuv-reporter gene are cloned into two compatible plasmid vectors, p5C and pJ61 (Supplementary Figure 1A), respectively. The vectors have been constructed using J61002 (BioBrick code BBa_J61002) as backbone with different antibiotic resistances (kanamycin and ampicillin, respectively) and origins of replication (ori: pBR322 and CloDF13, respectively). Among the two vectors, p5C contains a multiple cloning site (MCS) for the subcloning of two genes, each coding one of the split T7 RNAP fragments with possible fused domains and respective transcriptional terminators. The two genes are transcribed by a shared P_{cat} promoter (BioBrick code BBa_I14033) and translated with separate ribosome binding sites (RBS, BioBrick code BBa_B0034). The pJ61 plasmid contains the GFPuv gene[40] controlled by the T7 promoter. Detailed plasmid maps and oligonucleotides sequences are given in the Supporting Information.

To link the gene of a regulatory domain to the N-terminal (C-terminal) fragments of T7 RNAP, nucleotide sequences encoding the linker peptide EF-$(GS)_5$ ($(GS)_5$) were added to the N-terminus (C-terminus). The N-terminal fragment, with or without a regulatory domain at its C-terminus, was cloned into the p5C vector at the *Bgl*II/*Kpn*I sites. The C-terminal fragment, with or without a regulatory domain at its N-terminus, was cloned at the NdeI/SacII sites. To construct a nonsplit T7 RNAP with a Magnet domain inserted at position 564−565 (see Results and Discussion), a pair of *Eco*RI/*Bam*HI sites was introduced into the full-length T7 RNAP gene at this position and a Magnet domain flanked by respective linker sequences at both ends was cloned at the sites. Variants containing single-site mutations were obtained with site-directed mutagenesis performed according to the QuickChange protocol (Stratagene). All constructs have been verified by direct DNA sequencing.

Light Responsiveness Assays. Plasmids containing the modified T7 RNAP systems and the GFPuv reporter, respectively, were cotransformed into *E. coli* TOP10 competent cells and cultured with Ampicillin and Kanamycin added. Individual clones were picked and cultured in 3 mL of LB medium containing 100 μg/mL Ampicillin, 50 μg/mL Kanamycin, and 5 μM FAD at 37 °C overnight. The cells were

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ACS Synth. Biol. 2017, 6, 357−366

then 1% diluted in 6 mL of fresh medium and aliquoted into two tubes, one covered with tin foil to keep cells in dark conditions. Both tubes were placed in a shaking incubator at 250 rpm at 37 °C for 20 h with OD_{600} measured after incubation. Inside the incubator, the tubes remained to be illuminated with a common blue LED light. Cells with the same density (OD_{600}) were collected by centrifugation, washed, and suspended in 1 × PBS. Fluorescence was measured with a Spectra Max Gemini EM spectrophotometer with an excitation wavelength of 395 nm and an emission wavelength of 509 nm.

Time Course Analysis of Gene Expression. Transformed cells were cultured in LB medium containing respective antibiotics and FAD under dark conditions to mid log phase ($OD_{600} \approx 0.5$). Then cells were continuously grown in the dark or switched to light exposure for gene expression measurement in dark or light state, respectively. To investigate the process of activity turning off changing from the light condition to the dark condition, cells were placed in the dark after 5 h of light treatment. Samples were collected once per hour over a 14 h period. The fluorescence values at each time point were normalized by OD_{600} to provide fluorescence strength per cell.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acssynbio.6b00248.

Oligonucleotide primers used to obtain various T7 RNA polymerase gene fragments with flanking restriction enzyme sites (Supplementary Table 1). Oligonucleotide primers used to obtain genes for the "pure" fragments of T7 RNA polymerase (Supplementary Table 2). Oligonucleotide primers for site-directed mutagenesis to generate various VVD variants (Supplementary Table 3). Plamid sequences deposited in the GenBank database with accession numbers (Supplementary Table 4). Amino acid sequences of T7 RNA polymerase with the nine split positions indicated by red boxes (Supplementary Sequence 1). Amino acid sequences of the VVD domains with the linker sequences indicated by yellow boxes (Supplementary Sequence 2). Amino acid sequences of the GFPuv reporter (Supplementary Sequence 3). Schematic maps of various plasmids (Supplementary Figure 1). Growth curves of *E. coli* under light and dark conditions (Supplementary Figure 2). The scheme describing all possible dimerization options of the VVD domains and the crystal structure of VVD labeled with the positions of Cys71 and Cys108 (Supplementary Figure 3). Time-course analysis of the different light-inducible systems (Supplementary Figure 4). (PDF)

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Notes

The authors declare no competing financial interest.

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ACS Synth. Biol. 2017, 6, 357−366

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